(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 June 2006 (29.06.2006)

PCT

(10) International Publication Number
WO 2006/069000 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2005/045946

(22) International Filing Date:
20 December 2005 (20.12.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/637,664 21 December 2004 (21.12.2004) US
60/657,382 2 March 2005 (02.03.2005) US

(71) Applicant *(for all designated States except US)*: **THE BRIGHAM AND WOMEN'S HOSPITAL, INC.** [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **JAIN, Mukesh K.** [US/US]; 15 Grant Street, Newton, MA 02465 (US). **SENBANERJEE, Sucharita** [IN/US]; 1575 Tremont Street, Apt. 610, Boston, MA 02120 (US). **BANERJEE, Pallab** [IN/US]; 1575 Tremont Street, Apt. 610, Boston, MA 02120 (US). **LIN, Zhiyong** [CN/US]; 1575 Tremont Street, Apt. 1011, Boston, MA 02120 (US). **GARCIA-CARDENA, Gillermo** [MX/US]; 20 Chapel Street, #C708, Brookline, MA 02446 (US). **GIMBRONE, Michael A. Jr.** [US/US]; 30 Hopkins Road, Boston, MA 02130 (US).

(74) Agents: **SAMPLES, Kenneth H.** et al.; Fitch, Even, Tabin & Flannery, 120 South LaSalle Street, Suite 1600, Chicago, IL 60603 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: KLF2 AS A MEDIATOR OF STATIN ACTIVITY

(57) Abstract: The present invention is directed to methods for identifying compounds that mimic or inhibit the therapeutic effect of statins. The compounds are identified using assays of the transcription factor KLF2. The invention also includes methods for creating or identifying endothelial cells with low KLF2 activity which will be resistant to statin treatment and for increasing KLF2 activity by transfecting cells with expression vectors.

WO 2006/069000 A2

KLF2 as a Mediator of Statin Activity

Cross Reference to Related Applications

The present application claims priority to, and the benefit of, United States provisional applications 60/637,664, filed on December 21, 2004 and 60/657,382, filed on March 2, 2005. The contents of these prior applications are hereby incorporated by reference in their entirety.

Field of the Invention

The present invention is based upon the finding that statins cause their biological effects by increasing the activity of the KLF2 transcription factor. Thus, assays of KLF2 can be used to determine if a compound has statin-like activity or antagonizes statin activity. The invention also includes methods for identifying or creating endothelial cells with reduced KLF2 activity. These methods will be of interest to scientists studying disease processes affected by statins, and to clinicians interested in identifying individuals that are likely to be less responsive to statins and that may have a predisposition toward thrombus formation. One way of reducing this predisposition is to increase KLF2 activity by transforming the cells with vectors for expressing KLF2. Increasing KLF2 activity in endothelial cells may also be used as a means of inhibiting angiogenesis. Thus, agents that increase KLF2 activity have potential value in the treatment of solid tumors and will be of value to scientists studying tumor growth.

Background of the Invention

Clinical studies over the past decade have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A inhibitors (HMG-CoA reductase inhibitors, statins) can substantially reduce cardiovascular mortality. Although originally designed to reduce low-density lipoprotein levels, multiple lines of evidence suggest that the beneficial effects of statins exceed what may be anticipated by lipid lowering alone (Schonbeck U, *Circulation*. 109:II18-26 (2004); Liao, *et al.*, *Annu. Rev. Pharmacol. Toxicol.* (2004); Bellosta, *et al.*, *Ann. Med.* 32:164-76 (2000)). These favorable lipid-independent effects are thought to occur, at least in part, through alterations in vascular cell gene expression (Morikawa, *et al.*, *J. Atheroscler. Thromb.* 11:62-72 (2004)). For example, in endothelial cells, statins increase the accumulation of factors such as endothelial nitric oxide synthase

(eNOS) and thrombomodulin (Laufs, *et al.*, *Circulation 97*:1129-35 (1998); Laufs, *et al.*, *J. Biol. Chem. 273*:24266-71 (1998); Masamura, *et al.*, *Arterioscler. Thromb. Vasc. Biol. 23*:512-7 (2003)). The production of NO by eNOS is important not only for vasorelaxation but also has potent anti-inflammatory and anti-thrombotic properties (Vita, *et al.*, *Circulation. 102*:846-51 (2000); Landmesser, *et al.*, *Circulation 109*:II27-33 (2004); Mason, *et al.*, *Circulation. 109*:II34-41 (2004)). Thrombomodulin is a key endothelial cell surface factor that increases the rate of thrombin-catalyzed protein C activation (Esmon, *et al.*, *Proc. Natl. Acad. Sci. USA* 78:2249-52 (1981)). Furthermore, accumulating evidence suggests that TM impacts not only coagulation but also imparts anti-inflammatory, anti-thrombotic, and anti-adhesive properties to the endothelium (Esmon, *et al.*, *J. Endotoxin Res. 9*:192-8 (2003); Esmon, *J. Thromb. Haemost. 1*:1343-8 (2003)).

The molecular basis for how statins differentially regulate gene expression remains incompletely understood. Studies to date implicate small GTP binding proteins (*e.g.* Rho) and certain kinases (*e.g.* Akt) as important in mediating statin effects (Casey, *Science 268*:221-225 (1995); Zhang, *et al.*, *Annu. Rev. Biochem. 65*:241-69 (1996); Goldstein, *et* al, *Nature 343*:425-30 (1990); Fulton, *et al.*, *Nature. 399*:597-601 (1999); Liao, *J. Clin. Invest. 110*:285-288 (2002)). Much less is known about the nuclear mechanisms that maybe involved. A recent study suggests that statins diminish the activity/function of NF-κB and AP-1 – two key pathways regulating the induction of many pro-inflammatory genes (Dichtl, *et al.*, *Arterioscler. Thromb. Vasc. Biol. 23*:58-63 (2003)). However, what is very poorly understood are the nuclear mechanisms responsible for the ability of statins to induce target gene expression.

Summary of the Invention

Recently, a member of the Kruppel-like family of transcription factors termed KLF2 has been identified as a novel regulator of endothelial gene expression (SenBanerjee, *et al., J. Exp. Med.* 199:1305-15 (2004); Dekker, *et al.*, *Blood 100*:1689 (2002)). The present invention is based upon the discovery that statins exert their effects in endothelial cells by acting through this factor, apparently by inducing transcription from the KLF2 promoter. Thus, assays of KLF2 activity may be used to identify agents that should have the same beneficial therapeutic effects on endothelial cells as statins. These assays may also be used to identify factors that decrease KLF2 activity and which should

therefore antagonize the effects of statins. Identification of such factors is of importance because they have a potentially negative effect on therapeutic regimens involving statin administration. The invention also encompasses methods of identifying endothelial cells with low KLF2 activity or for making dysfunctional endothelial cells by engineering them in a manner that decreases KLF2 activity. Cells of this type will also be of interest in identifying new potential therapeutic agents and may be used by scientists examining factors that predispose cells to disease or that might make them resistance to certain therapies. Finally, the invention encompasses vectors for expressing KLF2 that may be used to transfect cells and generate a statin-like effect. Thus, cells transformed with the vectors should be, *inter alia*, more resistant to thrombosis.

In its first aspect, the invention is directed to an assay for determining if a test compound has either statin-like or statin-antagonizing activity. The term "statin-like" indicates that a compound has the same anti-inflammatory and anti-thrombotic effects on endothelial cells as the statins and this should be reflected in increased KLF2 induced production of eNOS and thrombomodulin. The term "statin-antagonizing" indicates that a compound inhibits KLF2 activity and should therefore reduce the effectiveness of statins. Cells with reduced KLF2 activity will require a higher dosage of statin to achieve the same therapeutic effect, or treatment involving the use of other agents that increase KLF2 activity.

The assays described above are performed by incubating test cells expressing KLF2 with the test compound and then determining the amount of KLF2 activity in the cells. Any method for assessing activity can be used, including determining the amount of KLF2 mRNA present, or by determining the amount of KLF2 protein present, *e.g.*, using an immunoassay. The results obtained from the test cells are compared with the results from control cells that are incubated in the absence of the test compound. Methods for selecting appropriate control cells are well known in the art. If the results of the comparison indicate that the incubated test cells have higher KLF2 activity than the control cells, it may be concluded that the test compound has statin-line activity. In contrast, if it is found that the test cells have less KLF2 activity than the control cells, it may be concluded that the test compound has statin-antagonizing activity.

It has been found that statins increase KLF2 activity by acting at the KLF2 promoter to increase transcription. Thus, assays which measure the rate of transcription from this promoter provide another way of determining whether a test compound has statin-like or statin-antagonizing activity. One preferred method for carrying out such assays involves first creating an expression vector in which the KLF2 promoter is operably linked to a marker gene. Appropriate marker genes are well known in the art and typically are selected for ease of detection and quantitation. Host cells are then transfected with the expression vector and incubated in the presence of the test compound. Expression of the marker gene is measured and the results are compared with those of control cells incubated in the absence of the test compound. An increase in marker gene expression is indicative of a test compound that acts in a statin-like manner, and a decrease in marker gene expression is indicative of a statin-antagonizing compound.

The invention also includes methods for creating dysfunctional endothelial cells by engineering them to reduce KLF2 activity. This can be done, for example, by using homologous recombination to disrupt the KLF2 promoter or the KLF2 structural sequence. The most preferred method however is to transfect the endothelial cells with a small interfering RNA (SiRNA) that decreases KLF2 activity. Methods for selecting an appropriate RNA molecule may be based upon the known KLF2 promoter and structural sequences using methodology that has been described in the art (see, *e.g.*, Gonzalez, *et al.*, *J. Biol. Chem. 279*:40659-40669 (2004); Gong, *et al.*, *Trends Biotechnol. 22*:451-454 (2004); Reynolds, *et al.*, *Nat. Biotechnol. 22*:326-330 (2004); Bertrand, *et al.*, *Methods Mol. Biol. 288*:411-430; Gilmore, *et al.*, *J. Drug Target 12*:315-340 (2004)).

In another aspect, the invention is directed to a method for identifying statin-antagonizing endothelial cells characterized by an abnormally low level of KLF2 activity. These cells should be less responsive to treatment using statins and patients having cells of this type may therefore require higher doses of statins or, alternatively, treatment that includes the use of other inducers of KLF2. The assay method involves comparing the KLF2 activity in selected endothelial cells with the amount of activity in a control population of cells. The control population may be endothelial cells derived from individuals known to respond to statins in the normal way or they may simply be cells derived from the general population. Any method for determining KLF2 activity is

compatible with the assay method, including assays for determining either mRNA levels or protein levels.

The invention also includes methods for increasing statin-like activity in cells by increasing KLF2 activity. This can be accomplished by transforming cells, especially endothelial cells, with an expression vector in which there is a promoter operably linked to a sequence coding for KLF2. By increasing KLF2 expression, the endothelial cell proliferation necessary for angiogenesis is inhibited. Any condition that can benefit by preventing the growth of new blood vessels can be treated using these methods, but it is expected that they will be of particular benefit in the treatment of solid tumors.

As discussed in the Examples section, the inhibition of endothelial cell proliferation by KLF2 appears to be attributable to amino acids 118-150 of KLF2 as shown in SEQ ID NO:1 and separately as SEQ ID NO:5. The invention includes peptides having this sequence, as well as substantially pure polynucleotides that contain a sequence encoding the peptides, and which can be used in their recombinant production. In addition, the invention encompasses vectors containing these polynucleotides, preferably operably linked to a promoter, and host cells transformed with the vectors. The term "substantially pure" refers to polynucleotides (or polypeptides) that have been separated from other accompanying biological components. Substantially purified molecules will typically constitute at least 80% of a sample, with greater percentages being preferred. Many means are available for assessing the purity of a protein or nucleic acid within a sample, including analysis of polyacrylamide gel electrophoresis, chromatography and analytical centrifugation.

The peptide described above may be used to inhibit the expression of VEGFR2 in endothelial cells. It may therefore be used for the purpose of inhibiting cell proliferation and, ultimately, angiogenesis. As with all of the methods described above, an effective amount of the inhibitory agent, in this case a peptide, must be brought in contact with the cells. The term "effective amount" refers to sufficient inhibitory agent to significantly reduce, *e.g.*, by at least 20%, the amount of cell proliferation and new blood vessel formation that would occur in the absence of the agent. In addition, it will be recognized by those of skill in the art that it is often possible to make minor changes in a peptide or

protein sequence and still maintain biological activity. For example, making conservative amino acid substitutions (*e.g.*, one acidic amino acid for another) is usually possible. Similarly an amino acid or two can be added or deleted with a high likelihood that activity will be maintained.

Brief Description of the Drawings

Figure 1: Figure 1 shows a protocol used to assess the effect of control adenovirus (Ad-GFP) and KLF2 adenovirus (Ad-K2) on VEGF-mediated angiogenesis in 6-8 week old nude mice. GFP= green fluorescent protein. K2=KLF2.

Figure 2: Figure 2 shows photographs of nude mouse ears before (-VEGF) and after (+VEGF) treatment with VEG-A in the presence (Ad-K2) and absence (Ad-GFP) of adenoviral KLF2. The dark blush after VEGF treatment (upper panels) in the control virus (Ad-GFP) treated ears represents the presence of new blood vessels. In contrast, a very minimal degree of blush is noted in the lower, KLF2 treated ears. The results suggest that KLF2 inhibits VEG-A-mediated angiogenesis.

Detailed Description of the Invention

I. Definitions

In order to provide a clear and consistent understanding of the present invention, the following definitions are provided:

Vector: The term "vector" refers to vehicles that can be used for transferring nucleic acid sequences into a host cell. Vectors are typically plasmids or viruses, such as retroviruses, adenoviruses, etc.

Expression vector: This refers to a vector which is capable of inducing the expression of DNA that has been cloned into it after transformation into a host cell. The cloned DNA is usually placed under the control of (*i.e.*, operably linked to) a promoter. Promoter sequences may be constitutive, inducible or repressible.

Host cell: As used herein, the term "host cell" refers to any prokaryotic or preferably eukaryotic cell that is the recipient of a vector. The term encompasses cells that

have been engineered to incorporate a gene into their genome, as well as cells that maintain transferred nucleic acid outside of the genome. Cells that can serve as hosts as well as techniques for cellular transformation are well known in the art (see, *e.g.*, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)).

Promoter: A "promoter" is a DNA sequence that initiates the transcription of a gene. For the purposes of the present invention, the most important promoter has the sequence shown as SEQ ID NO:3.

Expression: As used herein, the term "expression" refers to the process by which a polypeptide is produced from DNA. The process involves the transcription of a coding sequence into mRNA and the translation of this mRNA into a polypeptide. Depending on the context in which it is used, "expression" may refer to the production of mRNA, protein, or both.

Operably linked: The term "operably linked" refers to genetic elements that are joined in such a manner that enables them to carry out their normal function. For example, a gene is operably linked to a promoter when its transcription is under the control of the promoter and such transcription produces the protein normally encoded by the gene.

Gene: As used herein, "gene" refers to a nucleic acid sequence that undergoes transcription as the result of promoter activity.

KLF2: As used herein, the term "KLF2" refers to the human transcription factor that has been described in the art as having the sequence shown as SEQ ID NO:1 (Wani, *et al.*, *Genomics 60*:78-86 (1999); NCBI accession no. AF134053). It is encoded, *inter alia*, by the DNA sequence shown as SEQ ID NO:2 .

II. Assay Methods

Proteins and genes used in assays may be obtained using techniques well known in the art, or can be synthesized using chemical procedures. Guidance concerning sources

for all other factors needed in assays may be found in the Examples section, which also provides specific guidance concerning the way in which assays may be performed.

III. Construction and Use of KLF2 Expression Vectors

As discussed above, the invention includes recombinant DNA molecules in which there is a promoter, typically a mammalian promoter, operably linked to a sequence coding for the KLF2 protein as shown in SEQ ID NO:1. Procedures for obtaining promoters and other DNA sequences are well known in the art and standard techniques in molecular biology can be used for constructing DNA molecules with appropriately arranged elements (see, *e.g.*, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)).

Any type of promoter active in mammalian cells, and particularly endothelial cells, can be used in the invention, including those that are inducible, repressible or constitutive. In transcription assays designed to identify factors that have either statin-like or statin-antagonizing activity, the KLF2 promoter will be used (see, *e.g.*, SEQ ID NO:3). Expression vectors which are designed to increase KLF2 activity in cells may utilize any strong promoter which is active in the cells. For example, the human CMV immediate-early promoter (Boshart, *et al.*, *Cell 41*:521-530 (1985)) may be used. Standard vectors for expressing genes in mammalian cells are well known in the art and may be used in conjunction with the present invention.

When carrying out *in vitro* experiments, vectors may be introduced into cells using procedures such as calcium phosphate precipitation, microinjection, electrophoration, liposomal transfer, etc. When transfers are done to host cells *in vivo*, preferred methods of transformation are by means of a viral vector, liposomal transfer, or through the use of naked DNA. Cells that have incorporated constructs can be identified by assaying for the expressed product, using hybridization techniques, or by using the polymerase chain reaction (PCR) to amplify specific recombinant sequences.

IV. Inhibition of Angiogenesis

As discussed further in the Examples section agents that increase the expression of KLF2 in endothelial cells inhibit cell proliferation and angiogenesis. Thus, the vectors for

expressing KLF2 described above may be used in treating solid tumors and other conditions where blocking the growth of new blood vessels has a beneficial therapeutic effect.

It has also been concluded that the amino acid sequence corresponding to residues 118-150 of SEQ ID NO:1 is responsible for KLF2's inhibitory effect on endothelial cell proliferation. This sequence may be synthesized as a 33 amino acid peptide using standard chemical techniques and administered to patients to inhibit angiogenesis. The dosage administered to a patient can be determined using methods that are well known in pharmacolgy. The peptide may be administered in any phsiologically acceptable form including as a pharmaceutically acceptable salt. It may be included as part of a pharmaceutical composition and administered by any route that does not result in the destruction of peptide activity.

V. Protein and Nucleic Acid Sequences

The structure of human KLF2 is shown in SEQ ID NO:1. This protein as well as a peptide about 33 amino acids in length derived from it (SEQ ID NO:5) may be used to inhibit angiogenesis in a patient or in an experimental animal (see Figures 1 and 2). Polynucleotides encoding KLF2 or the anti-angiogenesis peptide can be constructed based upon a knowledge of the genetic code and used either therapeutically or to recombinantly produce polypeptide. Many methods are available for accomplishing this such as those described in Sambrook, *et al.* (Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press (1989)). However, it is preferred that both nucleic acids and polypeptides be made using standard methods of chemical synthesis.

VI. Therapeutic Methods

Therapeutic methods may involve either the administration of an anti-angiogensis polypeptide (*i.e.*, KLF2 or the peptide of SEQ ID NO:5) or the administration of nucleic acids that encode the polypeptide. In the latter case, oligonucleotides designed for the expression of polypeptide may be administered directly to patients. The *in vivo* transfection of cells has been known for many years and may be accomplished using viral vectors (see *e.g.* U.S. 6,020,191); liposomes (see *e.g.*, Nicolau, *Meth. Enzymol 149*:157-176 (1987)); DNA complexed to agents that facilitate cellular uptake (see *e.g.*, U.S.

5,264,618; WO 98/14431); or even by simply injecting naked DNA (see *e.g.*, U.S. 5,693,622). Administration may be repeated as is necessary until a positive therapeutic effect is observed. For example, DNA may be injected directly into or near a tumor until the growth of new blood vessels is retarded or until tumors diminish in size. Administration may be continued thereafter based upon clinical considerations.

As an alternative to gene therapy, polypeptide may be directly administered to a patient. For example the polypeptide may be injected into or near a tumor. In general, the polypeptide should be administered parentally, with administration by injection being preferred. The dosage administered to a patient will be determined by the attending physician based upon clinical considerations and using methods well known in the art.

Anti-angiogenesis polypeptides may be administered in either a single or multiple dosage regimen and may be given either alone or in conjunction with other therapeutic agents. Parenteral compositions may be used for intravenous, intraarterial, intramuscular, intraperitoneal, intracutaneous, or subcutaneous delivery. These preparations may be made using conventional techniques and may include isotonic saline, water, polyglycols, Ringer's solution, etc. Topical compositions may also be useful in treating cancers of the skin. All dosage forms may be prepared using methods that are standard in the art (see e.g., Remington's Pharmaceutical Sciences, 16 edition, A. Oslo editor, Easton, PA (1980)).

VII. Utility

The use of statins as therapeutic agents is well known in the art of clinical medicine. In addition to their known value in the treatment of cardiovascular conditions and inflammation, it has been suggested that statins may also be useful in blocking angiogenesis to prevent tumor growth, and in treating arthritis, osteoporosis, and stroke. Since the induction of KLF2 is the mechanism by which statins act, other inducing factors should have a similar therapeutic effect. Thus, the assays described herein may be used to identify potential therapeutic agents that act like statins. The assays may be based upon a measurement of KLF2 protein, or mRNA. Alternatively, they may measure the ability of test compounds to induce transcription from the KLF2 promoter, the specific mechanism by which the statins appear to increase KLF2 activity.

The assays are also useful in identifying agents that reduce KLF2 activity. Agents of this type should typically be avoided by patients undergoing treatment with statins. In addition, such compounds may be of interest to researchers studying thrombosis and attempting to identify the exact biological effects of anti-thrombotic drugs. In this regard, the ability to create endothelial cells with reduced KLF2 activity will also be useful.

Methods for assaying endothelial cells to determine whether they have normal levels of KLF2 activity will be useful for the reasons alluded to above. Specifically, patients having cells with reduced activity will typically require higher doses of statins to achieve a desired therapeutic effect. People with abnormally low levels of KLF2 may also be predisposed to development of conditions that respond to statin therapy, particularly cardiovascular diseases. Thus, assays of KLF2 activity in endothelial cells may provide a way of identifying individuals in need of preventive therapy, *i.e.*, treatment with an agent that raises KLF2 activity.

For the reasons discussed above, any agent known in the art that increases KLF2 activity in endothelial cells should be of potential clinical value as a therapeutic agent. One suitable agent is an expression vector for KLF2. In addition to having potential value in gene therapy methods, these expression vectors will provide researchers with a means for engineering cells *in vitro* that can be used to help to better define the biological effects of statins and endothelial cell pathologies.

Examples

Example 1: KLF2 as a Mediator of Statin Activity

A. Summary

The present example provides evidence that statin induced expression of eNOS and TM is KLF2 dependent. KLF2 mRNA was induced by treatment with multiple statins in a concentration-dependent manner. This effect was inhibited by both mevalonate and geranylgeranylpyrophosphate but not by farnesylpyrophosphate. Mevastatin induces KLF2 promoter activity through a single MEF binding site and mutation of this site abrogates the inductive effect. Finally, siRNA mediated knockdown of KLF2 strongly

attenuates the ability of mevastatin to increase eNOS and TM accumulation in endothelial cells.

B. Materials and Methods

Cell Culture and Reagents— Human umbilical vein endothelial cells (HUVECs) were acquired from Cambrex Bioscience (Walkersville, MD) and cultured in EBM-2 media according to manufacturer's instructions. All statins were purchased from Calbiochem and prepared according to manufacturer's recommendations. The thrombomodulin and MEF2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the eNOS antibody was from BD Biosciences (Palo Alto, CA); the α-tubulin antibody was from Sigma (St. Louis, MO). The adenoviral constructs were generated by the Harvard Gene Therapy Initiative (Boston, MA). The MEF2A and MEF2C plasmids and the –1.7kB-KLF2-Luc promoter were gifts from colleagues. All deletion constructs of the KLF2 promoter were generated by PCR and cloned into the PGL2 basic vector. Mutation of the MEF site was accomplished by using the QuikChange mutagenesis kit following the manufacture's instruction (Stratagene).

Northern and Western blot analysis — Cellular protein was extracted in RIPA buffer and Western blot analyses performed using the indicated antibodies as previously described (SenBanerjee, *et al.*, *J. Exp. Med.* 199:1305-15 (2004)). HUVECs were infected with Ad-GFP and Ad-GFP-KLF2 for 24 hours and then harvested for total protein analysis. Total RNA was obtained by using Trizol following the manufacturer's instruction and northern blot studies were performed as previously described (SenBanerjee, *et al.*, *J. Exp. Med.* 199:1305-15 (2004)). The KLF2 and KLF6 cDNA fragments were generated by RT-PCR.

Transient transfection assays — HUVEC cells were plated at a density of $5x10^4$/well in twelve well plates one day before transfection. A total of 0.3 μg of the indicated plasmid DNA was transiently transfected using Fugene™ 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to instructions by the manufacturer. Cells were treated with mevastatin 24 hours after transfection, harvested 48 h after

transfection, and assayed for luciferase activity normalized with β-galactosidase activity in each sample. For all transfections n=6-9.

Gel-shift Studies— The MEF site within the KLF2 promoter was used for gel-shift studies. The wild-type sequence was 5'-CCAGGCTTATATACCGCGGCTAAATTT AGGCTGAGCCCGGA-3' (SEQ ID NO:3). The mutant competitor sequence was 5'-CCAGGCTTATATACCGCGGCT**ATCGGT**AGGCTGAGCCCGGA (SEQ ID NO:4). MEF2A and MEF2C protein were generated by *in vitro* transcription and translation (Promega). Gel shift experiments were carried out as previously described (SenBanerjee, *et al.*, *J. Exp. Med.* 199:1305-1315 (2004)).

siRNA mediated knockdown studies— Small interfering RNA oligonucleotides were purchased from Dharmacon (Lafayette, CO) and knockdown performed as previously described with minor modifications (Gonzalez, *et al.*, *J Biol Chem.* 279:40659-69 (2004)). HUVECs were plated one day before transfection in antibiotic-free EBM-2 medium. On the day of transfection, 100nM of specific siRNA targeting human KLF2 or non-specific siRNA was incubated with Lipofectamine 2000 (Invitrogen) at room temperature for thirty minutes before adding to the HUVECs in OPTI-MEM (Invitrogen). Three hours later the medium was replaced by EBM-2 and cultured for an additional 48 hours. Cells were treated with or without mevastatin for 24 hours and harvested for protein.

C. Results

Effect of mevastatin and KLF2 on endothelial gene expression —

Previous studies indicate that statins can induce factors such as eNOS and TM (Laufs, *et al.*, *Circulation 97*:1129-35 (1998); Hernandez-Perera *J. Clin Invest. 101*:2711-2719 (1998)). Consistent with these observations, we found that treatment of human HUVECs with mevastatin (10 μmol/L) induced eNOS and TM protein levels.

Adenoviral overexpression of KLF2 in HUVECs alters the expression of endothelial products such as eNOS (SenBanerjee, *et al.*, *J. Exp. Med.* 199:1305-1315 (2004)) and TM (unpublished observation). By comparison to control infected cells (Ad-

GFP), adenoviral overexpression of KLF2 (Ad-GFP-K2) robustly induced both eNOS and TM protein levels.

Statin-mediated induction of KLF2 expression is dependent on cholesterol synthesis —

The effect of several statins on KLF2 mRNA expression was assessed and it was found that mevastatin, simvastatin, and lovastatin all induced KLF2 mRNA expression in HUVECs. In contrast, no significant effect was seen with pravastatin. The specificity of this inductive effect was verified by the fact that expression of KLF6, another member of this family known to be expressed in the endothelium, was not significantly altered by mevastatin. Furthermore, the induction of KLF2 by mevastatin occurred in a dose – dependent manner.

As a consequence of their ability to inhibit HMG-COA reductase, statins cause cells to be depleted in mevalonate. To test whether the statin-mediated induction of KLF2 expression was specific and dependent on mevalonate depletion, HUVECs were incubated with mevastatin in the presence or absence of mevalonate. It was found that supplementation with mevalonate completely blocked statin-dependent induction of KLF2.

Mevalonate is a precursor for cholesterol as well as isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGP). The isoprenoids are important post-translational lipid modifications of a variety of proteins such as Ras and Rho. Ras proteins are predominantly farnesylated whereas the Rho proteins are mainly geranylgeranylated. To test whether Ras or Rho have a role in the statin-dependent induction of KLF2, HUVECs were incubated with mevastatin in the presence of the isoprenoid intermediates FPP and GGPP. GGPP was found to partially reverse the mevastatin-mediated induction of KLF2 mRNA. In contrast, no effect was seen with FPP.

Statins induce the KLF2 promoter via a MEF binding site—

To gain greater insight regarding the molecular basis for statin-mediated induction of KLF2, we assessed the effect of these agents on KLF2 promoter activity. It was found

that mevastatin treatment induced the –1.7kB-Luc KLF2 promoter activity about three-fold. Using a series of deletion mutants, we found that the majority of the inductive effect was maintained using the –221bp-Luc promoter fragment but lost with further deletion to the -114bp-Luc construct. Thus, treatment with statins induces the KLF2 promoter through a critical 107 bp region.

Through close inspection of this region of the KLF2 promoter, we identified a single consensus MEF binding site. This site is typically bound by members of the MADS-box family of transcriptional regulators that are key regulators of muscle development. Interestingly, two members of this family, MEF2A and MEF2C, have also been implicated as regulators of endothelial cell biology (Wang, *et al.*, *Science 302*:1578-81 (2003); Lin, *et al.*, *Development 125*:4565-74(1998); Hayashi, *et al.*, *J. Clin. Invest. 113*:1138-48 (2004. To assess the importance of this site in statin-mediated induction of the KLF2 promoter, we mutated this region in the context of the –221-bp-Luc construct. It was found that mutation of the MEF site completely prevented the mevastatin-mediated induction of the KLF2 promoter. To determine if the MEF site was able to bind MEF proteins, we performed gel mobility shift assays. This confirmed that both MEF2A and MEF2C are able to strongly bind to this region of the KLF2 promoter. The specificity of binding was verified by competition and supershift studies. These data implicate downstream nuclear events mediated via MEF as being operative in the statin-mediated induction of the KLF2 promoter.

Effect of KLF2 "knockdown" on endothelial gene expression —

To determine if KLF2 is required for statin mediated effects, we undertook siRNA mediated knockdown studies (Elbashir, *et al.*, *Nature 411*:494-498 (2001)). The results obtained showed a strong reduction in KLF2 mRNA expression can be achieved with specific siRNA. The lack of a specific human KLF2 antibody precluded verification of the knockdown at the endogenous protein level. This effect was maximal by 48 hours and sustained for 72-96 hours. Next, HUVECs were transfected with non-specific or specific siRNA for 48 hours followed by treatment with mevastatin for 24 hours. It was found that, in the absence of statin treatment, the basal level of eNOS protein was reduced by approximately 37%. Furthermore, the approximate 2.3-fold induction of eNOS protein by mevastatin was completely abrogated following knockdown of KLF2. Similarly, an

approximate 72% reduction in TM levels was noted under basal conditions following knockdown of KLF2. In addition, while treatment with mevastatin induced TM levels of about 2.2-fold in the non-specific siRNA treated cells, only a 1.3-fold induction was noted after KLF2 knockdown. Taken together, these data support a critical role for KLF2 in mediating mevastatin's ability to induce endothelial gene expression.

D. Discussion:

The central findings of this study are that statins induce KLF2 expression and that a reduction in KLF2 expression attenuates statin-mediated accumulation of eNOS and TM levels. Our data also implicate another family of transcription factors – the MEFs – as being involved in the induction of KLF2. Taken together, these observations provide a novel pathway by which statins may exact favorable effects independent of lipid lowering.

Conditions that predispose to atherosclerosis such as diabetes, hypercholesterolemia, and hypertension are associated with endothelial dysfunction. As a consequence the endothelial phenotype is altered to one that is pro-adhesive and pro-thrombotic. Basic and clinical observations strongly support the ability of statins to improve endothelial health through differential effects on the expression of certain factors. In this regard, one of the most important effects of statins is to increase eNOS and TM levels in endothelial cells. This is thought to occur through both transcriptional and post-transcriptional means. Studies from our laboratory indicate that KLF2 can induce eNOS and TM mRNA and promoter activity suggesting that transcription events underlie, at least in part, the ability of this factor to induce these targets. However, this does not exclude the possibility that the KLF2 induction of eNOS and TM accumulation in endothelial cells may also be, in part, through post-transcriptional effects as well as effects on protein synthesis and stability. Indeed, the requirement for KLF2 in the ability of mevastatin to induce eNOS and TM strongly suggest that additional mechanisms are likely involved.

Our results also demonstrate that multiple statins – namely mevastatin, simvastatin, and lovastatin — can all induce KLF2. In contrast no effect was seen with pravastatin probably because the uptake of this compound is poor in endothelial cells. We also found that the induction of KLF2 by mevastatin was dependent on inhibition of cholesterol synthesis.

An intriguing observation made in this study is that mevastatin increases KLF2 promoter activity. These data support a link between the statin mediated inhibition of cholesterol/Rho activation and a transcriptional event that culminates in the induction of KLF2 expression. Our promoter deletion studies suggest that the transcriptional mediator(s) inducing the KLF2 promoter likely bind within a specific 104 base pair region (from –114 to > -221).

Example 2: KLF2 in Angiogenesis

The present Example describes evidence indicating that the induction of KLF2 leads to an inhibition of VEGF-mediated angiogenesis.

A. Background

A primary strategy in cancer treatment has been to block the development of the blood vessels which tumors need to sustain their growth. Angiogenesis requires the proliferation of endothelial cells, and this proliferation is induced by vascular endothelial growth factor (VEGF). The main receptor responsible for mediating the effect of VEGF in promoting endothelial cell proliferation is VEGFR2.

VEGF is also a pro-inflammatory agent and inflammation is considered a key feature in pathologic angiogenesis. VEGF treatment of HUVECs is known to induce key adhesion molecules (VCAM-1), coagulant proteins (tissue factor), and other factors (*e.g.*, COX-2). These factors have been implicated in a number of malignancies such as lung cancer and breast cancer. The combination of VEGF's pro-proliferative and pro-inflammatory effects contributes significantly to tumor angiogenesis.

B. KLF2 Regulates Endothelial Cell Proliferation

Studies were performed in which human umbilical vein endothelial cells (HUVECs) were transformed with adenovirus encoding either a control factor (GFP) or KLF2, both in the presence and absence of VEGF. It was found that KLF2 overexpression inhibits the increase in calcium flux and endothelial cell proliferation observed following VEGF treatment. Furthermore, results indicated that overexpression of KLF2 prevents the VEGF mediated induction of pro-inflamamtory targets such as VCAM-1, TF, and COX-2.

C. KLF2 Inhibits VEGFR2 Expression and Promoter

Adenoviral overexpression of KLF2 was found to reduce VEGFR2 expression, whereas the expression of VEGFR1 (a receptor believed to be involved in inhibiting angiogenesis) is slightly induced. A series of promoter deletion and mutational analyses were performed to identify the molecular basis of this effect. Based upon these studies and conclusions drawn using a sequence derived from a similar region of mouse KLF2 (SEQ ID NO:6), it was concluded that the amino acid sequence of SEQ ID NO:5 is able to inhibit VEGFR2 expression and, as a consequence, angiogenesis.

D. KLF2 Inhibits VEGF Mediated Angiogenesis *In Vivo*

Animals were injected with control (Ad-GFP) or KLF2 adenovirus (Ad-KLF2) and then exposed to VEGF-A (see Figure 1 for protocol). It was found that adenoviral overexpression of KLF2 prevented the VEGF-mediated induction of angiogenesis (see Figure 2). This was verified by staining for blood vessels.

What is Claimed is:

1. An assay for determining if a test compound has statin-like activity or statin-antagonizing activity, comprising:
   a) incubating test cells expressing KLF2 with said test compound;
   b) determining the amount of KLF2 activity in the incubated test cells of step a);
   c) comparing the results obtained in step b) with the KLF2 activity of control cells incubated in the absence of said test compound;
   d) concluding that said test compound has statin-like activity if the KLF2 activity determined for said test cells is greater than for said control cells; and
   e) concluding that said test compound has statin-antagonizing activity if the KLF2 activity determined for said test cells is less than for said control cells.

2. The assay of claim 1, wherein said test cells are endothelial cells.

3. The assay of claim 2, wherein said assay is performed by measuring the amount of KLF2 mRNA present in said test cells.

4. The assay of claim 3, wherein said assay is performed by reverse transcribing cellular KLF2 mRNA and then amplifying the product by the polymerase chain reaction (PCR).

5. The assay of claim 2, wherein said assay is performed by measuring KLF2 protein levels.

6. A method of determining whether a test compound has statin-like activity or statin-antagonizing activity, comprising assaying said test compound for its ability to promote transcription from the KLF2 promoter.

7. The method of claim 6, wherein said assay is performed by a procedure comprising:

a) creating an expression vector in which said KLF2 promoter is operably linked to a marker gene;

b) transfecting test cells with said expression vector;

c) incubating the transfected test cells of step b) with said test compound;

d) measuring the expression of said marker gene in the incubated cells of step c);

e) comparing the results obtained in step b) with the results from control cells incubated in the absence of said test compound;

f) concluding that said test compound has statin-like activity if the expression of said marker gene determined for said test cells is greater than that determined for said control cells; and

g) concluding that said test compound has statin-antagonizing activity if the expression of said marker gene determined for said test cells is less than that determined for said control cells.

8. A method for creating dysfunctional endothelial cells comprising engineering said endothelial cells to reduce KLF2 activity.

9. The method of claim 8, wherein said KLF2 activity is reduced by transfecting said endothelial cells with SiRNA for KLF2.

10. A method of identifying endothelial cells which have statin-antagonizing activity, comprising:

a) measuring KLF2 activity in said endothelial cells;

b) comparing the results obtained in step a) with the results obtained from assays of KLF2 activity performed using control cells;

c) concluding that said endothelial cells have statin antagonizing activity if the KLF2 activity in said endothelial cells is lower than the KLF2 activity in said control cells.

11. The method of claim 10, wherein said assay is performed by measuring the amount of KLF2 mRNA present in said endothelial cells.

12. The assay of claim 11, wherein said assay is performed by reverse transcribing cellular KLF2 mRNA and then amplifying the product by PCR.

13. The method of claim 10, wherein said assay is performed by measuring KLF2 protein levels.

14. A method of generating statin-like activity in cells, comprising transforming said cells with a nucleic acid that increases KLF2 expression.

15. The method of claim 14, wherein said cells are endothelial cells.

16. The method of claim 14, wherein said nucleic acid comprises a promoter active in mammalian cells, operably linked to a sequence coding for KLF2 protein.

17. A method of inhibiting the proliferation of human endothelial cells, comprising treating said endothelial cells with an effective amount of an agent that increases KLF2 expression in said cells.

18. The method of claim 17, wherein said agent is an expression vector encoding human KLF2.

19. A method of inhibiting angiogenesis in a patient, comprising administering to said patient an effective amount of an agent that increases KLF2 expression in endothelial cells.

20. The method of claim 19, wherein said agent is an expression vector encoding human KLF2.

21. A peptide comprising the amino acid sequence of SEQ ID NO:5, wherein said peptide inhibits VEGFR2 expression in endothelial cells.

22. The peptide of claim 21, wherein said peptide consists of the amino acid sequence of SEQ ID NO:5.

23. A substantially pure polynucleotide comprising a sequence encoding the peptide of either claim 21 or claim 22.

24. A vector comprising the polynucleotide of claim 23.

25. A host cell transformed with the vector of claim 24.

26. A method of inhibiting the expression of VEGFR2 in human endothelial cells, comprising contacting said cells with an effective amount of the peptide of either claim 21 or claim 22.

27. A method of inhibiting angiogenesis in a patient, comprising administering to said patient a therapeutically effective amount of the peptide of either claim 21 or claim 22.

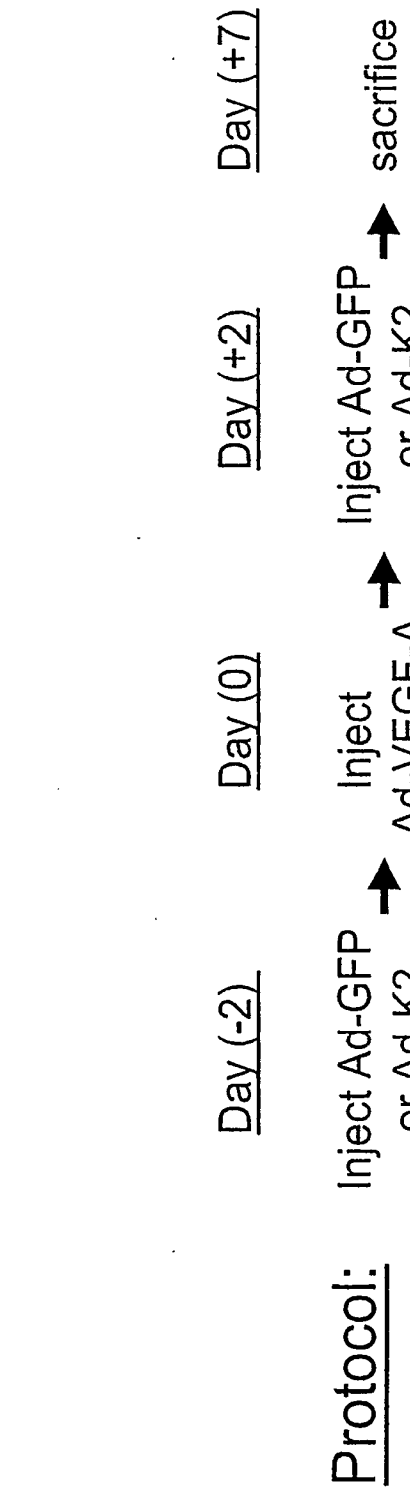

Figure 1

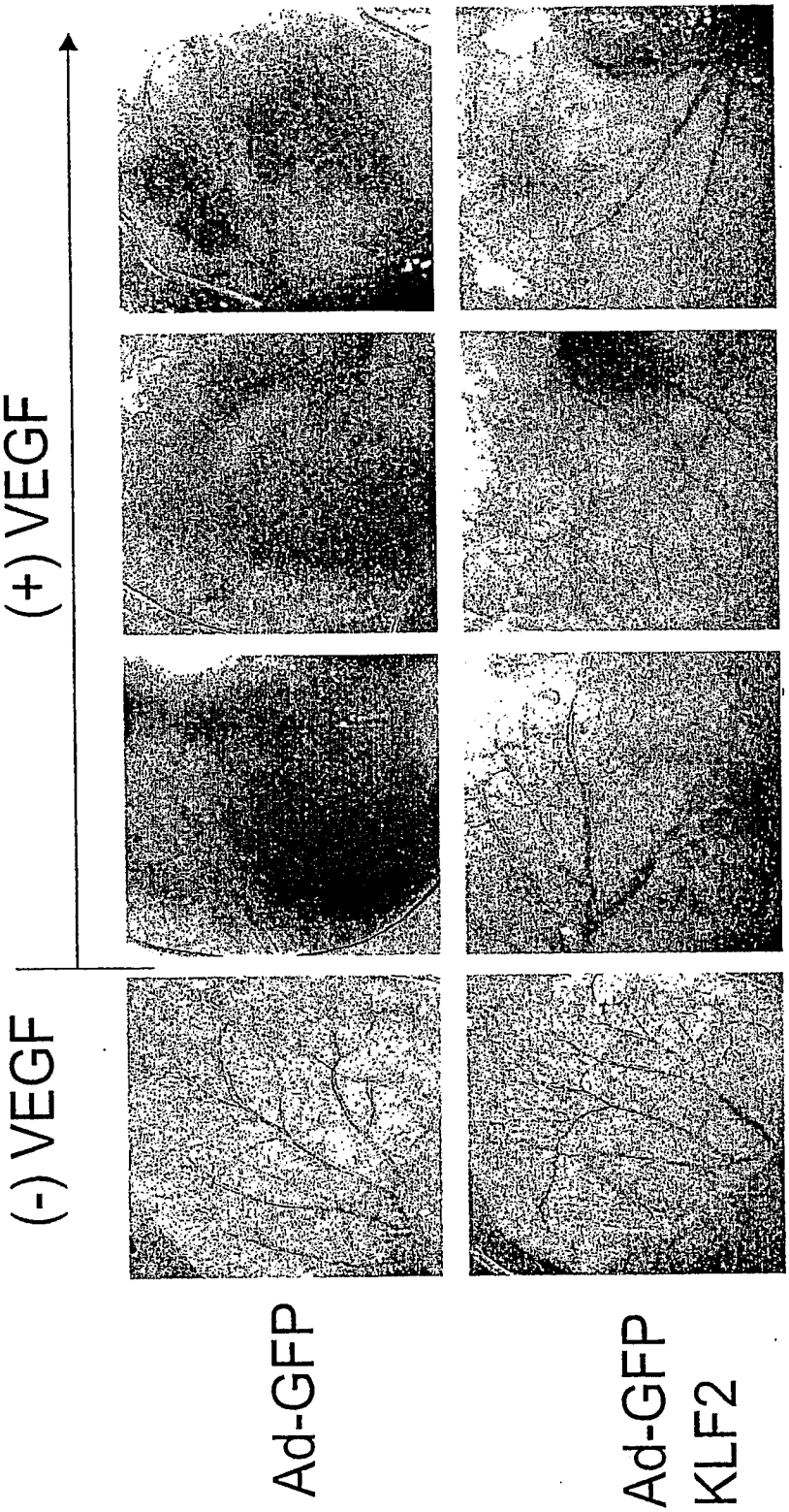

Figure 2

SEQUENCE LISTING

<110> The Brigham & Women's Hospital, Inc.
Jain, Mukesh
SenBanerjee, Sucharita
Banerjee, Pallab
Lin, Zhiyong
Guillermo Garcia-Cardena
Michael A. Gimbrone, Jr.

<120> KLF2 as a Mediator of Statin Activity

<130> 7570/84212

<160> 6

<170> PatentIn version 3.2

<210> 1
<211> 355
<212> PRT
<213> Homo sapiens

<400> 1

Met Ala Leu Ser Glu Pro Ile Leu Pro Ser Phe Ser Thr Phe Ala Ser
1 5 10 15

Pro Cys Arg Glu Arg Gly Leu Gln Glu Arg Trp Pro Arg Ala Glu Pro
20 25 30

Glu Ser Gly Gly Thr Asp Asp Asp Leu Asn Ser Val Leu Asp Phe Ile
35 40 45

Leu Ser Met Gly Leu Asp Gly Leu Gly Ala Glu Ala Ala Pro Glu Pro
50 55 60

Pro Pro Pro Pro Pro Pro Pro Ala Phe Tyr Tyr Pro Glu Pro Gly Ala
65 70 75 80

Pro Pro Pro Tyr Ser Ala Pro Ala Gly Gly Leu Val Ser Glu Leu Leu
85 90 95

Arg Pro Glu Leu Asp Ala Pro Leu Gly Pro Ala Leu His Gly Arg Phe
100 105 110

Leu Leu Ala Pro Pro Gly Arg Leu Val Lys Ala Glu Pro Pro Glu Ala
115 120 125

Asp Gly Gly Gly Gly Tyr Gly Cys Ala Pro Gly Leu Thr Arg Gly Pro
130 135 140

Arg Gly Leu Lys Arg Glu Gly Ala Pro Gly Pro Ala Ala Ser Cys Met
145 150 155 160

Arg Gly Pro Gly Gly Arg Pro Pro Pro Pro Pro Asp Thr Pro Pro Leu
165 170 175

Ser Pro Asp Gly Pro Ala Arg Leu Pro Ala Pro Gly Pro Arg Ala Ser
180 185 190

Phe Pro Pro Pro Phe Gly Gly Pro Gly Phe Gly Ala Pro Gly Pro Gly
195 200 205

Leu His Tyr Ala Pro Pro Ala Pro Pro Ala Phe Gly Leu Phe Asp Asp
210 215 220

Ala Ala Ala Ala Ala Ala Ala Leu Gly Leu Ala Pro Pro Ala Ala Arg
225 230 235 240

Gly Leu Leu Thr Pro Pro Ala Ser Pro Leu Glu Leu Leu Glu Ala Lys
245 250 255

Pro Lys Arg Gly Arg Arg Ser Trp Pro Arg Lys Arg Thr Ala Thr His
260 265 270

Thr Cys Ser Tyr Ala Gly Cys Gly Lys Thr Tyr Thr Lys Ser Ser His
275 280 285

Leu Lys Ala His Leu Arg Thr His Thr Gly Glu Lys Pro Tyr His Cys
290 295 300

Asn Trp Asp Gly Cys Gly Trp Lys Phe Ala Arg Ser Asp Glu Leu Thr
305 310 315 320

Arg His Tyr Arg Lys His Thr Gly His Arg Pro Phe Gln Cys His Leu
325 330 335

Cys Asp Arg Ala Phe Ser Arg Ser Asp His Leu Ala Leu His Met Lys
340 345 350

Arg His Met
355

<210> 2
<211> 1647
<212> DNA

<213> Homo sapiens

<400> 2
gcccgcccgc gccccgacca gcccggcctc gggcagccac tcaccggtgt ccccgtccgc 60

gtccttcctc cccgggtccc ggccatggcg ctgagtgaac ccatcctgcc gtccttctcc 120

actttcgcca gcccgtgccg cgagcgcggc ctgcaggagc gctggccgcg cgccgaaccc 180

gagtccggcg gcaccgacga cgacctcaac agcgtgctgg acttcatcct gtccatgggg 240

ctggatggcc tgggcgccga ggccgccccg gagccgccgc cgccgccccc gccgcctgcg 300

ttctattacc ccgaacccgg cgcgcccccg ccctacagcg cccccgcggg tggcctggtg 360

tctgagctgc tgcgacccga gctggatgcg ccgctggggc ccgcactgca cggccgcttt 420

ctgctggcgc cgcccggccg cctggtcaag gccgagcccc ctgaagcgga cggcggcggc 480

ggctacggct gcgcccccgg gctgacccgt ggaccgcgcg gcctcaagcg cgagggcgcc 540

ccgggcccgg cggcttcgtg catgcgaggt cccgggggcc gccccccgcc gccgcccgac 600

acaccgccgc tcagccccga cggccccgcg cgcctgcccg cgcccggtcc gcgcgcctcc 660

ttcccgccgc ctttcggtgg ccctggtttc ggcgcgcccg ggcccggcct gcattacgcg 720

ccgcctgcgc ccccagcctt cggtctcttc gacgacgcgg ccgccgccgc ggcagccctg 780

ggcctggcgc cccccgccgc ccgcggtctc ctcacgccgc ctgcgtcccc gctggagctg 840

ctggaggcca agccaaagcg cggccgccgc tcttggcccc gcaaacgcac cgccactcac 900

acctgcagct acgcgggctg cggcaagacc tacaccaaga gttcgcatct gaaggcgcat 960

ctgcgcacgc acacaggtga gaagccctac cactgcaact gggacggctg cggctggaag 1020

tttgcgcgct cagacgagct cacgcgccac taccgaaagc acacgggcca ccggccattc 1080

cagtgccatc tgtgcgatcg tgccttttcg cgctccgatc acctggcgct gcacatgaaa 1140

cggcacatgt agccgggacg cccccgccca cctgcggcgg gccgtggcgg gtcccacgcg 1200

ccgggcgcgg ccccctccca aactgtgact ggtatttatt ggacccagag aaccgggccg 1260

ggcacagcgt ggctacagag ggtctccctc gatgacgacg acgacgacgc caccacccca 1320

gcccccgtct gtgactgaag gcccggtggg aaaagaccac gatcctcctt gacgagtttt 1380

gtttttcaaa atggtgcaat aatttaagtg gcatcttctc tcccaccggg tctacactag 1440

aggatcgagg cttgtgatgc cttgtgagaa ataagggcct taatttgtac tgtctgcggc 1500

atttttttata atattgtata tagtgactga caaatattgt attactgtac atagagagac 1560

aggtgggcat ttttgggcta cctggttcgt ttttataaga ttttgctggg ttggtttttt 1620

ttttaattaa aaagttttgc atctttt 1647

<210> 3
<211> 41
<212> DNA
<213> Homo sapiens

<400> 3
ccaggcttat ataccgcggc taaatttagg ctgagcccgg a 41

<210> 4
<211> 41
<212> DNA
<213> Homo sapiens

<400> 4
ccaggcttat ataccgcggc tatcggtagg ctgagcccgg a 41

<210> 5
<211> 33
<212> PRT
<213> Homo sapiens

<400> 5

Gly Arg Leu Val Lys Ala Glu Pro Pro Glu Ala Asp Gly Gly Gly Gly
1 5 10 15

Tyr Gly Cys Ala Pro Gly Leu Thr Arg Gly Pro Arg Gly Leu Lys Arg
20 25 30

Glu

<210> 6
<211> 32
<212> PRT
<213> Mus musculus

<400> 6

Gly Arg Leu Val Lys Ala Glu Pro Pro Glu Val Asp Gly Gly Gly Tyr
1 5 10 15

Gly Cys Ala Pro Gly Leu Ala His Gly Pro Arg Gly Leu Lys Leu Glu
20 25 30